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(54) Title: ORAL VACCINES

(57) Abstract

A complex of an immunogen with a carrier molecule and a method for presentation of the immunogen to mucosal epithelia of a host vertebrate in order to elicit a systemic, cellular, and/or mucosal immune response in the host vertebrate to the complex. The invention also provides processes for the production of the complex. Further the invention provides medicaments containing the complex as well as medicaments which additionally contain dietary molecules. The dietary molecules provides a means of selectively modulating the magnitude and/or type of immune response to the complex of the medicament. The invention provides means for inhibiting gonadal function in a mammal as well as for selectively modulating cellular immune response to a complex according to the invention.

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"ORAL VACCINES"

TECHNICAL FIELD

The present invention relates to the specific stimulation of serum and secretory antibodies through mucosal presentation of antigens.

BACKGROUND ART

A number of infections in mammals have sufficient deleterious effects on the mammal to warrant vaccination against the particular antigen responsible for the infection. Therefore, vaccination programmes are instituted whereby the mammal is antigenically challenged with an antigen so that an immune response is elicited to confer immunity to the mammal.

Administration of the antigen to the mammal may be through a number of routes including injection intramuscularly (i.m.), subcutaneously (s.c.), or through oral administration (per os). I.m. or s.c. injection of antigen suffers from the disadvantages that relatively specialized skills are required, it is difficult to undertake on a large scale, it is expensive, and a number of side reactions can occur to either the immunizing antigen or to the emulsifying reagent in which it is presented. Oral administration of vaccines is by contrast relatively problem free except insofar as oral feeding of a number of antigens requires relatively large quantities of antigen as the amount of material that is actually absorbed and capable of stimulating an effective immune response is usually low. Thus the amount of antigen required for oral immunization generally far exceeds that required for systemic induction of immunity. There is also one major disadvantage to the oral presentation of the large quantities of antigen required to produce an antibody response and that is that feeding of these large quantities of antigen often leads to the induction of systemic tolerance (Tomasi, 1980; Mowat, 1985; Mowat and Parrot, 1983; Ngan & Kind, 1978; Hanson et al, 1979; Richman et al. 1978; Rothberg et al., 1973).

Evidence to date suggests that in general the mechanism by which antigen is taken up by the small intestine, following oral feeding, is primarily via non-specific sampling of the contents of the gut lumen by "M" cells which overlie the Peyer's Patches and other lymphoid clusters of the GALT (gut-associated-lymphoid tissue) (Bland and Britton, 1984). The subsequent sensitization of local lymphocyte populations

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leads to the generation of local IgA immune responses plus the
sensitization of IgG suppressor cells with concomitant suppression of
serum IgG responses (Tomasi, 1980; Mowat, 1985; Mowat and Parrot,
1983; Ngan & Kind, 1978; Hanson et al, 1979; Richman et al, 1978;
5   Rothberg et al, 1973).

It is therefore apparent that the site of antigen uptake,
whether it be through the Peyer's Patches or the villous epithelium,
and quite probably the amount of antigen administered, dictates the
type of immune response generated by orally administered antigen. The
10.   question arises then as to whether there are any other antigens apart
from cholera toxin which exhibit the ability to specifically prime the
mucosal immune system upon oral challenge and/or to stimulate the
humoral immune response in a dose dependant manner without inducing
systemic tolerance and without the need for excessive doses of antigen.
15   With this view in mind we decided to investigate the possible
potential of certain adhesive molecules, which have been implicated in
the initial attachment of a number of intestinal pathogens, to
stimulate the immune response when orally administered. These surface
antigens which confer adhesive properties to a number of strains of
20   enterotoxigenic E. coli (ETEC) have been identified as nonflagellar,
filamentous proteinaceous appendages, or pili (Gaastra & de Graaf,
1982). Examples include the CFA I and CFA II antigens of human ETEC
strains and the K88, K99, F41 and 987P pili of animal ETEC strains
(Gibbons et al, 1975; Evans & Evans, 1978; Levine et al., 1980; Morgan
25.   et al., 1978; de Graaf & Roorda, 1982). In addition, we have examined
the ability of a number of other proteins which have no apparent role
in colonization to prime the immune system upon oral feeding. These
antigens included a number of lectins a serotypic antigen of
S.typhimurium (the type "i" flagella), inactivated flu virus and
30   S.typhimurium endotoxin (LPS). Oral priming was compared to the
response generated to wholly intramuscular challenge (i.m.).

Thus, the aim of these studies was to provide a method whereby
the uptake of an immunogen or antigen by the gastrointestinal tract
mucosa is improved to the extent that it is possible to elicit serum
35   and secretory antibodies by oral feeding of low doses of the immunogen
without the induction of oral tolerance.

Accordingly the invention describes a group of molecules

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(mucosal immunogens) which when fed lead to the production of serum antibodies to these proteins at comparable levels to those obtained by intramuscular injection of these molecules. Furthermore when larger quantities of these antigens are fed there is a concomitant stimulation of the production of mucosal antibodies to the immunizing molecules.

In a further aspect of this invention a process is described whereby the antibody response generated to the orally fed molecules can be augmented or changed by the co-feeding of a number of dietary molecules.

In another aspect of this invention a process is described whereby a hapten or protein can be coupled to a mucosal immunogen and the complex of which, when fed, results in the production of antibodies to the hapten or coupled protein.

ABBREVIATIONS

1.	Ab	-	Antibody
2.	BSA	-	bovine serum albumin
3.	ConA	-	Conconavlin A
4.	DNP	-	dinitrophenyl
5.	ELISA	-	Enzyme linked immunosorbent assay
6.	ETEC	-	enterotoxigenic E. coli
7.	GALT	-	gut associated lymphhoid tissue
8.	HA	-	hydroxy apatite
9.	im	-	intra muscular
10.	LHRH	-	luteinizing hormone releasing hormone.
11.	LPS	-	lipopolysaccharide
12.	LT-B	-	heat labile toxin of enterotoxigenic E. coli.
13.	O/N	-	overnight
14.	per oo	-	oral administration
15.	ps	-	polysaccharide
16.	RT	-	room temperature
17.	sc	-	subcutaneous
18.	SDS-PAGE	-	SDS - polyacrylamide gel electrophonesis
19.	TCA	-	trichloracetic acid.

DISCLOSURE OF INVENTION

In a first form the invention provides a complex comprising: an immunogen; linked to a carrier molecule which is capable of

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specifically interacting with the mucosal epithelium of a vertebrate
host; wherein both the immunological activity of the immunogen and the
capacity of the carrier molecule to specifically interact with the
mucosal epithelium of the vertebrate host is substantially maintained,
and said complex is capable of eliciting a systemic, cellular and/or
mucosal immune response in the vertebrate host.

Preferred immunogens according to the invention include:

all, part, analogues, homologues, derivatives or combinations
thereof and a hormone, thereapeutic agent, antigen or hapten. These
immunogens include hormones such as LHRH (luteinising hormone
releasing hormone) FSH, HGH and Inhibin; allergens such as grass
pollens (for instance barley and couch), weed pollens (eg. clover,
dock), tree pollens (eg. ash, cyprus), plant pollens (eg. broom),
epithelia (eg. cat hair, dog hair, pig hair) and house dust, wheat
chaff and Kapok; immunogens for vaccines against agents such as
influenza, measles, Rubella, smallpox, yellow fever, diphtheria,
tetanus, cholera, plague, typhus, BCG, haemophilus influenzae,
Neisseria catarrhalis, Kelbsiella pneumonia, pneumococci and
streptococci especially S. mutans; and pili including pili derived
from E. coli, N. gonorrhoeae, N. meningitis, N. catarrhalis, Yersinia
spp, Pseudomonas aeruginosa, Pseudomonas spp, Moraxella bovis,
Bacteroides nodosus, Staphylococci spp, Streptococci spp and
Bordetella spp.

Preferred carrier molecules include bacterial adhesins such as
987P, K99, CFAI, CFAII, K88 or F41; viral haemagglutinins such as from
influenza, measles, Rubella, smallpox or yellow fever viruses; toxins
or binding subunits thereof such as LTB ricin, abrin, diphtheria
toxin, modecin, tatanus toxcin and others of similar structures; and
lectins whether from plant or other origin. Lectins include for
example conconavalin A, Pokeweed mitogen or lectins from Lens
culinaris, Helix pomatia, Glycine max, Arachis hypogea, or Ulex
europeus or Abrin, Asparagus pea, Broad bean, Camel's foot tree,
Castor bean, Fava bean, Green marine algae, Hairy vetch, Horse gram,
Horse shoe crab, Jack bean, Japanese wisteria, Jequirity, Scotch
laburnum, Lima bean, Limulin, Lotus, European mistletoe, Mung bean,
Osage orange, Pagoda tree, Garden pea, Potato, Red kidney bean, Red
marine algea, Siberian pea tree, edible snail, garden snail, Spindle

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tree, Sweet pea, Tomato, wheat germ or winged pea.

In a preferred embodiment of the invention there is provided a complex which comprises luteinising hormone releasing hormone and LTB.

In another form the present invention provides a process for the production of a complex as described above which process comprises:

(a) reacting the immunogen with the carrier molecule to form said complex;

(b) chemically modifying the immunogen to provide at least one functional group capable of forming a chemical linkage, and reacting the immunogen and carrier molecule to form said complex; or

(c) chemically modifying the carrier molecule to provide at least one functional group capable of forming a chemical linkage, and reacting the immunogen and carrier molecule to form said complex;

(d) chemically modifying the immunogen and the carrier molecule to provide functional groups capable of forming a chemical linkage, and reacting the immunogen and carrier molecule to form said complex;

(e) reacting the immunogen with at least one linking agent, and reacting the immunogen and the carrier molecule to form said complex;

(f) reacting the carrier molecule with at least one linking agent and reacting the immunogen and the carrier molecule to form said complex;

(g) reacting the immunogen and the carrier molecule with at least one linking agent, and reacting the immunogen and the carrier molecule to form said complex; or

(h) a combination of any of the preceding process steps.

In another form the invention provides a process which comprises providing a recombinant DNA molecule comprising a first DNA sequence which on expression codes for the amino acid sequence of the immunogen, a second DNA sequence which on expression codes for the amino acid sequence of the carrier molecule, and vector DNA; transforming a host with said recombinant DNA molecule so that said host is capable of expressing a hybrid, proteinaceous product which comprises said complex; culturing said host to obtain said expression; and collecting said hybrid proteinaceous product.

Alternatively the invention provides a process for the production of a complex which process comprises

(a) chemically synthesising the immunogen and/or the carrier

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molecule, and forming the complex by chemical reaction;
or   (b)   synthesising a hybrid peptide comprising amino acid
sequences of the immunogen and the carrier molecule. Preferably the
peptide is prepared by solid phase, enzymatic or manual peptide
5   synthesis.

In a preferred form of the invention the synthesised immunogen
or carrier molecule whilst bound to the resin of the solid phase
peptide synthesiser is coupled to the carrier molecule or immunogen
respectively.

10   In another form of the present invention there is provided a
transformant host transformed with a recombinant DNA molecule
comprising a first DNA sequence which on expression codes for the
amino acid sequences of all, part, an analogue, homologue, derivative
or combination thereof, of the immunogen, a second DNA sequence which
15   on expression codes for the amino acid sequence of all, part, an
analogue, homologue, derivative or combination thereof of the carrier
molecule, and vector DNA.

In a preferred form of the invention the transformant host is a
Gram negative or Gram positive bacterium, a yeast, fungus or a higher
20   eukaryotic cell. In one preferred form of the invention, said host is
E.coli. A culture of a transformant microorganism falling within the
scope of the present invention had been deposited with the American
type culture collection and has been designated the number:

In a further form the invention provides a recombinant DNA
25   molecule comprising a first DNA sequence which on expression codes for
the amino acid sequence of the immunogen, a second DNA sequence which
on expression codes for the amino acid sequence of the carrier
molecule, and vector DNA. In a preferred embodiment of this form of
the invention, the vector DNA is plasmid DNA but alternative vectors
30   are envisaged within the scope of the present invention and these
include viruses, bacteriophages and cosmids. In one preferred form of
the invention there is provided plasmid pBTAK66 which when a host cell
is transformed with said plasmid will yield a proteinceous product
which includes a polypeptide falling with the scope of the present
35   invention.

In a further form of the invention there is provided a
polynucleotide sequence which comprises a first hybrid polynucleotide

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sequence which acts as a coding sequence for a fusion product
comprising an amino acid sequence of an immunogen fused to an amino
acid sequence of a carrier molecule, a polynucleotide sequence
sufficiently related to said first hybrid polynucleotide sequence so
5   as to hybridize to said first hybrid polynucleotide sequence, a
polynucleotide sequence related by mutation, including single or
multiple base substitutions, deletions, insertions and inversions, to
said first hybrid polynucleotide sequence or hybridizing sequence or a
polynucleotide sequence which on expression codes for a polypeptide
10 which displays similar biological or immunological activity to said
fusion product.

Preferably the polynucleotide sequence is one wherein the first
hybrid polynucleotide sequence acts as a coding sequence for the amino
acid sequence of all, part, an analogue, homologue, derivative or
15 combination thereof of LHRH fused to the amino acid sequence of a
carrier molecule, more preferably LTB.

In a further form of the invention there is provided a
medicament which comprises a complex according to the invention
together with a pharmaceutically acceptable carrier or diluent. .
20 Examples of pharmaceutically acceptable carriers and diluents include
typical carriers and diluents such as tablets, aqueos solutions,
sodium bicarbonate solutions and similar diluents which neutralise
stomach acid or have similar buffering capacity, glycols, oils,
oil-in-water or water-in-oil emulsions, and include medicaments in the
25 form of emulsions, gels, pastes and viscous colloidal dispersions.
The medicament may be presented in capsule, tablet, slow release or
elixir form or as a gel or paste or may be presented as a nasal spray
and in this form may be in the presence of an aerosol. Furthermore,
the medicament may be provided as a live stock feed or as food
30 suitable for human consumption.

The present inventors have also found that co-administration of
certain dietary molecules with a complex of the present invention can
selectively modulate the magnitude and/or type of the immune response
to the immunogen of the complex.

35 Accordingly the present invention further provides a medicament
which comprises the complex of the present invention together with a
dietary molecule which dietary molecule can selectively modulate the

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magnitude and/or type of the immune response to the immunogen of the
complex.

The dietary molecule envisaged by the present invention include
basic, neutral and acidic amino acids, such as argenine, histidine,
5   lysine, alanine, cysteine, cystine, glycine, isoleucine, leucine,
methionine, phenylalanine, proline, serine, threonine, tryptophan,
tyrosine, valine, aspartic acid, glutamic acid; water soluble and
insoluble vitamins, such as thiamine, riboflavin, pyridoxal,
cyanocobalamin ($V.B_{12}$) ascorbic acid (V.C). Vit D_2, etc -
10   Ergosterol, Vit.E, Vit.A, Vit K etc; sugars including monosaccharides
e.g. galactose, mannose, mannitol, sorbitol, glucose, xylose, Allose,
altrose, arabinose, digitoxose, erythrose, fructose, lyxose, muramic
acid, mannose, pyruvic acid, ribose, tagatose, talose and the amidated
and N acetylated, derivatives thereof; oligosaccharides e.g. lactose,
15   maltose, melibiose, sucrose, cellubiose, N,N diacetyl chitobiose,
gentobiose, isomaltose, lactobionic acid, trehalose, turanose; and
dietary minerals and co-factors such as manganese, magnesium, zinc,
calcium and iron.

The invention also provides a method of presenting a complex of
20   the present invention which method comprises the mucosal
administration of a complex of the present invention together with a
dietary molecule capable of modulating the magnitude and/or type of
immune response of the immunogen.

The invention also provides the oral administration of the
25   medicaments of the invention, in order to elicit a response to the
active molecule in the host. Such a response, in the case where the
active molecule is an antigen or hapten may be a systemic and/or a
mucosal immune response. In the case where the active molecule is
LHRH or a derivative, analogue, homologue, part or combination
30   thereof, of LHRH, the response will be inhibition of gonadal function
in the host. Where the oral medicament incorporates a dietary
molecule according to the invention, the invention provides a method
for enhancing the host's response to the active molecule which
comprises administering such an oral medicament to the host.

35   BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the N-terminal amino acid sequence of the 987P
pilin subunit, in comparison with the N-terminal amino acid sequence

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of other pilin proteins.

MODES FOR CARRYING OUT THE INVENTION

Materials

Lectins were purchased from Sigma Chemical Co. Inactivated flu
vaccine was purchased from the Commonwealth Serum Labs. (Australia).
Sugars and vitamins were obtained from the following sources:-
Lactose (AR grade) - Ajax Chemicals, Sydney, Australia; Fructose D(-),
Mannose D(+), Sorbitol and Xylose D(+) (all AR grade)- B.D.H.
Chemicals Ltd. Poole, England; Melibiose D(+)- Sigma Chemical Co., St.
Louis, Miss.; Retinal (Vit A. aldehyde)- Fluka AG, Chemicals, Fabrik
Buchs, Switzerland; Thiamine - HCl (Vit B1), Riboflavin (Vit B2),
Pyridoxal (Vit B6), Cyanocobalomin (Vit B12), L-ascorbic acid (Vit C),
Ergosterol (Pro Vit D) and dl-a-tocopherol (Vit E)- Sigma Chemical
Co., St. Louis, Miss.

Bacterial Strains and Media

The E. coli K99, 987P and LTB strins used in these experiments
are listed in Table 1, and were the generous gifts of Dr. Susan Clark,
(Molecular Biology Laboratory Biotechnology Australia). Cultures were
grown at 37C (unless otherwise specified) with shaking in Luria broth
(LB) with or without 1mM isopropylthio-n-D-galactopyranoside (IPTG) as
indicated (Table 1). Salmonella typhimurium was grown at 37C with
shaking in LB plus 0.2% glycerol.

TABLE 1

Stain	Genetic Marker	Strain Origin	Phenotype
BTA 595	p BTA 193	RB 791	LTB +
BTA 604	p BTA 201	MC 1061	987P
BTA 262	p BTA 106	ED 8654	K99

+ Included c̃ 1mM IPTG

PURIFICATION OF ANTIGENS

Pili preparation

E. coli expressing the cloned pili, as detected using
radiolabelled antiserum were harvested during logarithmic phase of

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growth. Cultures were heated at 60C for 30 minutes, after which the
organisms were pelleted by centrifugation (3,000 x g. 30 min 4C). The
supernatant was examined for pili content by 12.5% SDS-PAGE using a
modification of the method of Laemmli (Laemmli, 1970; Salit et al.,
5   1980).

        K99 purificaton:- The culture supernatant was adjusted to pH
9.7 with (10N NaOH and stirred at room temperature (R.T.) for 10
minutes. The resultant precipitate containing pili was recovered by
centrifucation (3,000 x g, 30 min 4C) and resuspended in 100ml
10   distilled H_2O (dH_2O) pH 7.2. This procedure was repeated twice.

        987P Purification:- Procedures used were as detailed above
except that pili precipitation was achieved by adjusting the pH to 3.9
with glacial acetic acid.

        Hydroxyapatite chromatography
15   Hydroxyapatite (HA) (DNA grade Bio-Gel HTP, Bio-Rad) was gently
swollen in an excess of dH_2O and after a brief period ($<$ 2 min.)
fines were decanted gently. Fresh dH_2O was added and used to gently
resuspend the gel after which fines were decanted again. This
procedure was repeated several times. A column (30 x 5 cm) was filled
20   with a slurry of approximately 30% HA and allowed to settle by
gravity. Tight packing was then achieved by passing dH_2O through
the column at a flow rate of 16ml/hr until the gel bed surface was
stationary. Samples (100 ml) of either K99 or 987P were appied at
flow rates not exceeding 30ml per hour. The column was then washed
25   with dH_2O until no protein was detected in the flow through as
detected at 280 nm. Pili were eluted at a flow rate of 30ml/hr using
a linear gradient of 15 to 250 mM sodium phosphate pH 7.5. Fractions
were collected and examined by SDS-PAGE. The pili peak was recovered
and pooled.

30   Ion-exchange Chromatography
        Pooled fractions of K99 and 987P pili (from the HA
chromatography) were reprecipitated with NaOH (pH 9.7) or glacial
acetic acid (pH 3.9) repsectively. After centrifugation (3,000 x g,
10 min), the pellets containing pili were resuspended in 50 mM Citrate
35   buffer, pH 5.5 (K99) and 50 mM Tris.HCl pH 8.5 (987P) prior to loading
on the ion-exchange columns equilibrated with the same buffers. K99
and 987P were loaded onto CM and DEAE columns respectively at a flow

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rate of 100ml/hr, washed with 2 volumes of loading buffer and the pili
eluted using a linear gradient from 10 mM to 0.5 M NaCl in the
aquilibration buffers. Fractions were examined by SDS-PAGE for
protein content and LPS contamination, according to the method of Tsai
.5   and Frasch (1982).

LTB Purification

Three litres of LTB supernatant was diluted to 6l with dH_2O.
The pH was adjusted to 6.5 with glacial acetic acid and loaded onto a
5 x 30 cm column of fastflow CM-Sepharose equilibrated with 10 mM
10   phosphate buffer pH 6.5 at a flow rate of 1.2 l/hr. The column was
then washed with 400 mls of 10 mM phosphate buffer pH 6.5 and bound
protein aluted with a linear gradient of 10-500 mM Nacl in 10 mM
phosphate pH 6.5 fractions were collected and analysed by SDS-PAGE,
the LTB peak was pooled.

15   Flagellae Isolation

Late log phase cultures of bacteria were pelleted by
centrifugation (3,000 x g for 15 mins. at 4C). The cells were
resuspended in saline and heated at 60C for 30 minutes, followed by
centrifugation (3000 x g, 10 min 4^oC). The supernatant was
20   precipitated by adding a solution of 100% TCA (w/v) to give a final
concentration of 10% (w/v) and spun for 10 min at 1,500 x g 4C. The
pellet was resuspended in a small volume of 1 M Tris pH 8.8 and
sonicated until in solution. Ethanol was added to a final
concentration of 80% (v/v) and the flagellae spun down at 2,000 x g,
25   10 min at 4C. The pellet was resuspended in acetone, sonicated into
suspension and reprecipitated by centrifugation (5,000 x g). Finally
the pellet was brought into solution by boiling in 10% SDS and 50 mM
EDTA in 10 mM Tris. HCl pH 8.0. prior to Sephacryl S-200
chromatography.

30   Flagellae purification

After boiling for 15 min the flagellae were clarified by
centrifugation for 5 min. in a Beckman benchtop microfuge to remove
non-solubilized material. The supernatant was applied to a 2.5 x 80
cm column of Sephacryl - S200 (Pharmacia, Fine Chemicals) equilibrated
35   with 20 mM Tris pH 8.8, 0.1% SDS and 10 mM EDTA and eluted using the
same buffer. Fractions were collected and analysed by SDS-PAGE.
Finally the flagellae peak was pooled and precipitated with 10% (final

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conc.) TCA followed by centrifuation, ethanol and acetone washes as
described previoulsy. The final pellet was resuspended in dH_2O.

Lipoplysaccharide (LPS) Purification

Overnight cultures of S. typhimurium were extracted (30 min
R.Temp) with 0.5 M $CaCl_2$ in 20% ethanol (v/v) containing 100 mM
citrate pH 3.0 and 5% Zwittergent 3,12 (w/v) (Calbiochem.). Bacteria
were pelleted by centrifugation (3,000 x g, 10 min at 4C) and the
pellet resuspended in 50 mM EDTA pH 8.0 The suspension was stirred
vigorously for 30 min at R.temp. After removal of the bacteria by
centrifugation ethanol was added to the supernatant to a final
concentration of 75%. Protein material was pelleted and the
supernatant adjusted to 90% ethanol. The precipitate which formed was
pelleted and washed with acetone, reprecipitated and finally
resuspended in dH_2O. The preparation was assayed for sugar content
using the Anthrone reagent (Herbert et al, 1985) and checked for the
presence of contaminating proteins using SDS-PAGE. Commercial E. coli
LPS (Sigma Chemical Co.,) was used as a standard in both assays. Gels
were stained for LPS using a silver stain according to the method of
Tsai and Frasch (1982).

Preparation of Polysaccharide (PS)

Lipid A was cleaved from the S. typhimurium LPS preparation by
incubating the LPS with 1 M glacial acetic acid and heating at 100C
for 2-5 hrs. Lipid A was then removed by centrifugation at 3,000 x g
for 10 mins at 4C.

Description of Purified Antigens

SDS-PAGE analysis of purified K99 and 987P pili preparations
revealed the presence of a single band migrating at 17,500 and 20,000
mol. wt. (respectively) under reducing conditions (Fig. 1). This
agrees with the published data of Isaacson and others (Isaacson &
Richter. 1981; Morris et al. 1980; de Graaf et al, 1981; Fusco et al,
1978). The ease of precipitation of these proteins at pH 9.7 and 3.9
(for K99 and 987P, respectively suggests that the pI of these two
proteins to be around these ranged (see:- Isaacson & Richter, 1981;
de Graaf et al, 1981). Silver staining of these preparations showed
them to contain little (< 1 µg / 100 µg protein) or no
contamination with LPS.

Determination of the 987P Amino Terminal Sequence

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Amino terminal micro-sequencing was perfored for us by
Biotechnology Research Enterprises S.A. Pty. Ltd. Adelaide, South
Australia. A 100 nmole sample of 987P purified as described above was
assayed. The amino terminal sequence of 987P is compared with the
.5   published sequence of K99 and reveals no homology between these two
molecules (Fig. 1) (Gaastra and de Graaf, 1982).

Purified LTB, and S.typhimurium flagellae were also found to be
free of contaminating LPS and to travel as monomers of apparent
molecular weights of 12,500 and 52,000 respectively when examined by
10   SDS-PAGE under reducing conditions.

Silver stained SDS-PAGE gels of purified LPS revealed no
detectable protein contamination. Complex sugar content, as assayed
by Anthrone reaction, was found to be 2mg/ml. Lipid A free
polysaccharide was also found to contain 2 mg/ml polysaaccharide and
15   it's failure to move on SDS-PAGE (as revealed in the silver stained
gels) showed it to be free of contaminating lipid.

Dinitrophenylation of Antigens

K99, LTB and lectins were dintrophenylated according to the
method of Little and Eisen (1967). Briefly, carriers (in 0.1 M
20   carb/bicarb buffer pH 9.5) were reacted with a 0.1 M solution of DNFB
(in Acetone) overnight at R.T. The proteins were then dialysed
extensively against the coupling buffer. Previous studies by us have
shown that 987P has no free amino groups exposed for coupling so a
diamino spacer was first linked to the free carboxyl moieties of the
25   protein as follows; 10 mg of purified 987P was precipitated at pH 3.9
by the addition of glacial acetic acid. The pili were removed by
centrifugation at 3,000 x g, 10 min at 4C. The pellet was resuspended
in dH_2O and the pH raised to 6.5 with IN NaOH. The pili solution
was then reacted with 1-ethyl-3- (3-Dimethylaminopropyl)
30   carbodiimide-HCL (EDAC, Bio Rad Laboratories, Richmond, California),
to a final concentration of 0.5 mM in the presence of 20 mM
1,2-diaminoethane (BDH Chemicals Ltd. Poole, England), overnight at
room temperature (20-23C). The amino-substituted 987P was dialysed
for 24 hrs against two changes of 0.1 M carbonate/bicarbonate buffer
35   pH 9.5 before being used in the subsequent conjugation steps. Lectin
binding sites were protected during their reaction with DNFB by the
addition of the Lectin specific sugars. Thus, 50 mM solutions of

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D-glucose, D-mannose, D-glucose, N-acetyl-D-galactosamine,
D-galactose, N-acetyl-D-galactosamine, D-gal(1-3)-D-galN-Ac, and
L-fucose, were added to the following lectins:- Conconavlin A,
Pokeweed mitogen, Lens culinaris, Helix pomatia, Phaseolus vulgaris
Glycine max, Arachis hypogea and Ulex eruopeus, respectively.

Antigen administration

Female C57BL/6J mice (18-22 gm) were obtained from the Aminal
Resources Centre (Perth, Western Australia). All mice were starved
for 3-4 hours prior to oral or intramuscular (i.m.) administration of
antigens. Mice were fed antigen at appropriate concentrations in 0.5
ml of 0.1M carb/bicarbonate buffer pH 9.5 usng a specially prepared
feeding needle. Parallel doses of antigen were injected i.m., in
0.1ml of sterile physiological saline, into the left hind leg. Groups
of 5 mice receiving antigen either orally or im were given two
identical doses of antigen, on day 0 and day 14. A blood sample was
taken (approx. 0.5 ml) from the retro-orbital plexus on day 14 and day
21. Mice were then sacrificed by cervical dislocation and gut washes
performed on the small intestine in the following manner. The small
intestine was carefully removed and a small quantity of washing buffer
(1.0 ml, 30 mM Tris.HCl pH 8.8, 0.9% NaCl, 50 mM EDTA plus 1.0% Tween
20) introduced into the lumen of the gut via a blunt ended feeding
needle. After gently kneading the intestine the contents were
squeezed out through forefinger and thumb. Gut washes so obtained
were immediately centrifuged to remove debris and stored at -20C until
assayed. Blood samples were allowed to clot at 4C before removal of
the serum and storage at -20.

Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA for the determination of antibody titres was performed
as described previously by Russell-Jones et al., (1984).

EXAMPLE 1

Identification of Molecules Active as Mucosal Immunogens

The possible potential of a number of molecules known to possess
the capacity to bind to the intestinal mucosa and to stimulate the
production of an immune response after oral administration of these
molecules was examined. The response generated by these molecules was
compared to the response seen after similar feeding of other molecules
having no mucosal binding functions.

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As seen in Table 1.1, three broad classes of proteins were detected in these experiments:- those that elicited a serum and intestinal response, K99, 987P, LTB, flu vaccine and the various lectins (Class 1) (these shall henceforth be referred to as mucosal immunogens), those that elicited only a serum response (LPS) (Class II) and those that failed to elicit either a serum or intestinal response at the doses tested (Flagellae, BSA and P.S.) (Class III). Within class I antigens 987P was a significantly superior stimulator of IgA antibody (ab) (48.5 ± 1.8) when compared to LTB (12.2 ± 4.4), or K99 (3.2 ± 4.9). In addition 987P also stimulated gastrointestinal IgG (10.8 ± 1.76) to a greater extent than either K99 (3.0 ± 5.3), or LTB (1.0), and only 987P was capable of stimulating serum IgA (10.8 ± 8.8). All four class I antigens stimulated serum IgG to similar degrees (Table 1.1). The class II antigen, LPS, stimulated a small serum IgG response (12.1 ± 1.0) with no concomitant IgA or agastrointestinal reactivity. Finally BSA, flagellae and the polysaccharide moeity of LPS - class III antigens - failed to induce either serum or intestinal IgG or IgA. Representative samples from all three classes; K99, 987P, LTB, LPS and flagellae were administered intramuscularly and screened for both serum and intestinal response (Table 1.2). Class I and II antigens gave similar serum IgG responses yet failed to produce serum IgA or intestinal IgG/IgA Ab responses. Only the anti-LPS serum IgG response appeared significantly improved by i.m. immunisation. Each of 987P, K99 and LTB were further examined in dose response studies after both oral and i.m. administration. As seen in Tables 1.3 and 1.4, 987P yielded consistently higher titers than either K99 or LTB regardless of the route of administration. Interestingly the class I antigen - LTB - displayed a bell shaped dose-response with a plateau maximum between 10 and 50 μg. None of the other class I antigens produced this effect, nor did the LTB when administered Im. Oral administration of all class I antigens (Ag) elicited higher levels of intestinal IgA Ab (S-IgA) over the broad range of doses tested. Comparison between the two routes of administration suggests that although i.m. injection consistently gave higher titers, oral administration of mucosal immunogens resulted in comparable levels of antibody production to that obtained by the i.m. route between 10 and 100 μg of antigen for class I and II antigens.

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Table 1.1

Immune reactions to orally presented antigens

Antigen used for immunisation (20 µg does)	Immune response,* day 21.			
	Serum		Intestinal	
	IgG	IgA	IgG	IgA
K99	968 ± 120	<4	3.0 ± 5.2	3.2 ± 4.9
987P	776 ± 64	10.8±8.8	10.9 ± 1.7	48.5 ± 1.88
LTB	1351 ± 211	<4	<4	12.2 ± 4.4
Flue vac	179 ± 34	<4	<4	<4
Flagellae	<4	<4	<4	<4
LPS	12.1 ± 1.0	<4	<4	<4
PS	<4	<4	<4	<4
BSA	<4	<4	<4	<4
Con A**	666 ± 84	<4	nd	nd
FW-mitogen**	641 ± 119	<4	nd	nd
L. culinaris**	954 ± 48	<4	nd	nd
H. pomatia**	591 ± 127	<4	nd	nd
P. vulgaris**	1378 ± 110	4.8 ± 2.3	nd	nd
G. max**	1529 ± 65	3.1 ± 6.9	nd	nd
A. hypogea**	1276 ± 242	<4	nd	nd
U. europeus**	1583 ± 94	<4	nd	nd

*   The reciprocal of the antiserum dilution that gave an ELISA reading of 0.5 after 45 minutes at 37C. Each value represents the mean value obtained from 5 mice ± 1 standard deviation.

**   Each lectin was substituted with 4 DNP molecules/mole lectin. The ELISA titre represents the anti DNP response as measured against DNP-BSA.

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Table 1.2

Immune reactions to antigens presented intramuscularly

Antigen used for immunisation (20 µg does)	Immune response,* day 21.			
	Serum		Intestinal	
	IgG	IgA	IgG	IgA
K99	1024 ± 94	<4	<4	<4
987P	1552 ± 112	<4	<4	<4
LTB	1782 ± 100	<4	<4	<4
Flagellae	1595 ± 227	<4	<4	<4
LPS	388 ± 58	<4	<4	<4

*   See Table 1.1

Table 1.3

Dose response to orally presented antigens

Antigen	Immune repsonse*, day 21, per dose (ug)					
	0.1	1.0	10	50	100	1,000
Serum IgG						
K99	1.0	4.0	28	675	1351	4096
987P	4.0	14	84	588	1024	3104
LTB	9.0	194	1351	1331	891	891
Serum IgA						
K99	<4	<4	<4	<4	<4	<4
987P	<4	<4	<4	9.6	18.3	32
LTB	<4	<4	<4	<4	<4	<4
Intestinal IgG						
K99	<4	<4	<4	<4	<4	<4
987P	<4	<4	<4	4.0	4.0	8.0
LTB	<4	<4	<4	<4	<4	<4
Intestinal IgA						
K99	<4	<4	<4	4.0	16.7	87
987P	<4	<4	9.1	54	84	147
LTB	<4	<4	<4	4.0	4.0	8.0

* see Table 1.1.

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Table 1.4

Dose response to intramuscularly presented antigens

Antigen	Immune response*, day 21, per dose (ug)					
	0.1	1.0	10	50	100	1,000
Serum IgG						
K99	256	445	776	1351	1776	5104
987P	256	891	1024	3104	4096	18820
LTB	64	588	1176	2702	4096	8192
Serum IgA						
TK99	<4	<4	<4	<4	<4	<4
987P	<4	<4	<4	<4	<4	<4
LTB	<4	<4	<4	<4	<4	<4
Intestinal IgG						
K99	<4	<4	<4	<4	<4	<4
987P	<4	<4	<4	<4	<4	<4
LTB	<4	<4	<4	<4	7.0	16
Intestinal IgA						
K99	<4	<4	<4	<4	<4	4.6
987P	<4	<4	<4	<4	4.0	6.0
LTB	<4	<4	<4	<4	16.0	11.1

* See Table 1.1.

EXAMPLE 2

Effect of dietary molecules on the immune response to mucosal immunogens upon oral presentation.

Initial studies in our laboratory using outbred Swiss male mice suggested that it was possible to alter the immune response to orally presented antigens by the co-feeding of certain dietary molecules. Accordingly, the mucosal immunogens, K99, 987P and LTB were fed to mice in the presence of a mumber dietary sugars and vitamins. It was reasoned that as the different antigens were known to bind to different molecules on the surface of the intestinal epithelium, and as it is known that there is a change in the distribution of glycoproteins and glycolipids throughout the length of the gut as well as a change in distribution of absorptive cells, it might be possible to stimulate the uptake of molecules bound to these cells by presenting the antigens in the presence of the specific dietary molecule normally taken up by these cells. An extension to this

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argument would be that the profile of stimulatory molecules would change from antigen to antigen.

The results represented in tables 2.1, 2.2 and 2.3 demonstrate that although most of the vitamins and sugars have had some effect in modulating the immune response to K99, 987P and LTB, some dietary molecules appear to be selective as to which mucosal immunogen they appeared to influence but also as to whether they induced primarily a secretary or serum response. Thus, the serum antibody response to K99 was significantly increased ($P<0.05$) by co-administration with Vit B12 of Melibiose, unchanged when given with Vit B2, Vit D, Vit E, Fructose or Mannose and decreased to varying extents with Vit A, Vit B1, Vit B6, Vit C, Lactose, Sorbitol and Xylose (Table 2.1).

In contrast, the serum Ab response to oral 987P (Table 2.2) was elevated when 987P was co-administered with Vit B6, Vit B12, Vit C, Vit E, Fructose or Mannose, it was unchanged with Vit A, Vit B1, Vit B2, Lactose, Melibiose, Sorbitol and Xylose, and decreased in the presence of Vit D. LTB on the otherhand, displayed a unique profile for the effect of co-feeding of dietary molecules on the serum Ab levels. The results in Table 2.3 clearly show an augmented serum titer to LTB in the presence of Vit A, Vit B2, Vit D, Fructose, Mannose and Xylose. Little or no change with Vit B1, Vit B12, Vit C or Melibiose and almost complete inhibition with Vit B6, Vit E, Lactose, Melibiose and Sorbitol. The inhibition of an immune response seen with Vit B6, Lactose, Melibiose and Sorbitol is to be expected due to the similarities in structure of these compounds to Galactose which is claimed to be the specific sugar determinant on the GM1 ganglioside to which LTB is known to bind. These results are broadly suggestive that K99, 987P and LTB bind to and are internalized by discreet cells of the microvillous epithelium.

Dose response experiments (Tables 2.4, 2.5 and 2.6) demonstrate that is possible to stimulate the secretary arm of the immune system without concomitant stimulation of serum antibodies, or conversely to augment the serum response without affecting the level of secretary Abs, by the simple addition of dietary molecules to the orally presented mucosal immunogens. Thus cofeeding of large doses of Vit B12 or melibiose with K99 leads to a two to eightfold (respectively) increase in serum Ab with little concomitant increase in secretory

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Abs. Conversely cofeeding of Vit D in increasing doses lead to a drop
in serum Abs and a rise in secretory Ab. Certain dietary molecules on
the other hand also result in stimulation of both secretory and serum
Ab titres as shown by an eightfold increase in serum Ab and a 1000
fold increase in S-IgA upon cofeeding of Vit C with 987P.

     Experiments in which the mucosal immunogens were injected i.m.
together with vitamins or sugars showed little effect if any on the
immune response thus demonstrating that the change in response due to
cofeeding of these molecules with the mucosal immunogens must occur on
or near the site of absorption of these molecules rather than directly
upon the immune system (Table 2.7).

TABLE 2.1

Effects of dietary molecules on the immune response to orally
administered K99 (20μg)*

Dietary	Dose	Antibody Response			
Molecule		Serum		Intestinal	
		IgG	IgA	IgG	IgA
none	-	968±120	<4	3.0±5.2	3.2±4.9
Vit A	20μg	278±184	3.4±4.7	1.5±1.1	5.4±3.0
Vit B1	"	117±107	1.5±2.0	2.7±1.0	2.1±0.9
Vit B2	"	604±216	<4	2.3±1.7	2.0±0.6
Vit B6	"	14±50	<4	2.0±1.5	3.5±8.2
Vit B12**	"	3377±1266	4.0±3.0	<4	<4
Vit C**	"	318±255	2.0±2.8	32 ±1.1	98±70
Vit D**	"	1921±640	<4	<4	6.3±2.7
Vit E	"	512±128	<4	<4	4.4±2.1
Fructose	50mM	1782±966	8.4±3.7	2.9±1.6	34.7±14.2
Lactose	"	84±204	<4	<4	22.9±6.7
Mannose	"	1176±411	2.6±3.4	10.2±3.4	21.1±40.3
Melibiose**	"	1840±208	1.2±1.4	3.2±2.0	4.4±3.7
Sorbitol	"	77±179	<4	1.3±0.4	20.5±3.4
Xylose	"	328±217	<4	1.9±1.1	2.8±1.3

* The reciprocal of the antiserum dilution that gave an ELISA
reading of 0.5 after 45 min at 37°C on day 21 after immunisation.
Each value is the mean of five mice ±1 standard deviation.

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** Each value is the mean of 15 mice ±1 standard deviation. These molecules were also tested in dose response experiments.

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TABLE 2.2

Effects of dietary molecules on the immune response to oral 987P (20µg)*

| | | Antibody Response | | | |
| | | Serum | | Intestinal | |
Dietary Molecule	Dose	IgG	IgA	IgG	IgA
none	-	776±64	10.8±8.8	10.9±1.7	48.5±1.8
Vit A**	20µg	648±40	29.0±20	8.4±3.1	20.5±24:6
Vit B1	"	891±127	12.1±3.6	9.4±1.5	14.7±5.5
Vit B2	"	1082±271	31.1±11.9	17.8±7.6	21.1±11.9
Vit B6	"	1782±509	23.6±8.7	32.8±9.3	39.9±7.1
Vit B12	"	3983±1307	48.5±16.0	35.7±3.4	91.7±31.9
Vit C**	"	4521±1046	54.2±19.2	10.8±6.9	84.1±14.3
Vit D**	"	398±89	18.8±11.0	13.1±4.9	34.7±8.1
Vit E	"	3468±776	9.18±2.6	11.1±4.2	19.9±3.9
Fructose	50mM	2048±894	10.8±3.6	15.1±4.7	23.5±3.2
Lactose	"	1128±662	15.3±2.9	19.6±2.5	21.7±6.1
Mannose	"	4970±2270	24.9±11.7	14.3±1.9	16.9±26.4
Melibiose**	"	1243±474	30.3±7.7	91.7±10.9	124.5±22.6
Sorbitol**	"	1389±307	38.8±19.1	22.9±4.6	91.7±18.6
Xylose	"	1024±941	6.2±1.9	19.6±7.1	21.4±5.4

* See Table 2.1
** See Table 2.1

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TABLE 2.3

Effects of dietary molecules on the immune response to oral LTB (20μg)*

Dietary Molecule	Dose	Antibody Response			
		Serum		Intestinal	
		IgG	IgA	IgG	IgA
none	-	1351±211	<4	<4	12.2±4.4
Vit A	20μg	4705±676	<4	<4	21.1±7.6
Vit B1	"	1782±309	<4	<4	16.0±2.1
Vit B2	"	3565±908	<4	<4	16.0±3.7
Vit B6**	"	9.18±1.1	<4	<4	<4
Vit B12	"	1024±116	<4	<4	24.2±11.9
Vit C	"	337±206	<4	<4	16.1±5.0
Vit D	"	4097±74	<4	<4	13.9±2.2
Vit E	"	194±64	<4	<4	18.4±3.6
Fructose	50mM	6208±1192	<4	<4	10.5±3.3
Lactose**	"	5.0±1.0	<4	<4	<4
Mannose	"	4096±658	<4	<4	24.2±8.1
Melibiose	"	512±76	<4	<4	8.0±4.0
Sorbitol**	"	8.0±1.2	<4	<4	<4
Xylose	"	5404±2211	<4	<4	21.1±9.2

*   See Table 2.1

** See Table 2.1

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TABLE 2.4

Effect of orally administered dietary molecules on the immune
response to oral antigen (K99)*

Dietary Molecule	Day 21 Immune response per dose (μg~, mM+)						
	0.1	1.0	10	50	100	500	1000
Serum IgG							
Vit B12	256	675	1176	1552	2352	nd	2352
Vit C	512	891	588	675	1024	nd	891
Vit D	588	588	776	891	1782	nd	891
Melibiose	128	256	675	4096	9410	256	nd
Serum IgA							
Vit B12	<4	<4	4.0	5.2	<4	nd	<4
Vit C	<4	<4	4.0	16.0	16.0	nd	16.0
Vit D	<4	<4	<4	<4	<4	nd	<4
Melibiose	<4	<4	<4	4.0	4.0	4.0	nd
Intestinal IgG							
Vit B12	<4	<4	<4	<4	<4	nd	<4
Vit C	<4	4.0	16.2	32.0	36.1	nd	38.0
Vit D	<4	<4	<4	<4	<4	nd	<4
Melibiose	<4	<4	<4	4.0	4.0	4.5	nd
Intestinal IgA							
Vit B12	<4	<4	<4	<4	5.2	nd	13.9
Vit C	<4	<4	4.0	16.0	36.7	nd	73.5
Vit D	<4	<4	4.0	8.0	13.9	nd	24.2
Melibiose	5.2	4.0	4.0	4.0	9.2	4.0	nd

~, dose in μg, vitamins; +, dose in mM, sugars.

* See Table 2.1

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TABLE 2.5

Effect of orally administered dietary molecules on the immune
response to oral antigen (987P)

Day 21 Immune response per dose (ug~,mM+)

Dietary molecule	0.1	1.0	10	50	100	500	1000
Serum IgG							
Vit A	512	675	388	891	675	nd	588
Vit C	891	1351	4096	8192	8192	nd	9410
Vit D	2352	2048	256	73	147	nd	1351
Melibiose	512	481	463	1250	1006	1292	nd
Sorbitol	873	1133	1024	1400	2123	85	nd
Serum IgA							
Vit A	<4	<4	16.0	30.0	36.0	nd	64.0
Vit C	4.0	4.0	32.0	66.0	73.1	nd	86.7
Vit D	64	16.0	16.0	16.0	28.0	nd	38.1
Melibiose	nd	nd	nd	nd	nd	nd	nd
Sorbitol	nd	nd	nd	nd	nd	nd	nd
Intestinal IgG							
Vit A	<4	<4	4.6	17.2	16.1	nd	16.0
Vit C	<4	<4	16.0	6.0	5.2	nd	16.0
Vit D	<4	<4	<4	12.2	14.6	nd	14.0
Melibiose	5.0	27	75	76	38.3	4.3	nd
Sorbitol	50	74	64	93	294	257	nd
Intestinal IgA							
Vit A	<4	<4	4.0	26.9	32.1	nd	16.0
Vit C	<4	4.6	55	337	1024	nd	776
Vit D	<4	<4	16.0	147	25.2	nd	36.7
Melibiose	7.6	39	65	159	58.0	16.0	nd
Sorbitol	52	51	67	111	178	180	nd

~, dose in ug, vitamins; +, dose in mM, sugars

* see Table 2.1

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TABLE 2.6

Effect of orally administered dietary molecules on the
immune response to oral antigen (LTB)*

Day 21 Immune response per dose (ug~,mM+)

Dietary molecule	0.1	1.0	10	50	100	500	1000
Serum IgG							
Vit B6	2352	5048	55.7	10.5	4.6	nd	4.0
Lactose	1782	1782	73.5	6.9	4.0	4.0	nd
Sorbitol	1024	256	18.3	13.9	4.0	4.0	nd
Serum IgA							
Vit B6	<4	<4	<4	<4	<4	nd	<4
Lactose	<4	<4	<4	<4	<4	<4	nd
Sorbitol	<4	1.4	<4	<4	<4	<4	nd
Intestinal IgG							
Vit B6	<4	<4	<4	<4	<4	nd	<4
Lactose	<4	<4	<4	<4	<4	<4	nd
Sorbitol	<4	<4	<4	<4	<4	<4	nd
Intestinal IgA							
Vit B6	5.6	<4	<4	<4	<4	nd	<4
Lactose	4.0	4.0	<4	<4	<4	<4	nd
Sorbitol	4.0	<4	<4	<4	<4	<4	nd

~, dose in ug, vitamins; +, dose in mM, sugars

* see Table 2.1

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TABLE 2.7

Effect of varying the dose of co-administered dietary molecules
on the immune response to intramuscularly presented antigen K99, 987P*

Day 21 Immune response

Antigen given	Dietary molecule						
	(ug)	0.1	1.0	10	50	100	1000
(s) IgG							
K99	Vit B12	1121	1468	1572	2328	4766	2109
K99	Vit D	1024	1272	1168	1372	1489	1315
987P	Vit C	1687	1529	1707	1700	1662	1891
	(mM)	0.1	1.0	10	50	100	500
(s) IgG							
K99	Melibiose	1024	1176	1057	1392	1262	989
987P	Melbiose	1538	1622	1701	1519	1666	1621
987P	Sorbitol	1670	1577	1548	1632	1711	1651

+. no serum IgA, intestinal IgG or intestinal IgA was detected
* See Table 2.1

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EXAMPLE 3

The two previous examples demonstrated that small doses of orally administered mucosal immunogens have the capacity to induce significant serum IgG titers with or without a parallel rise in secretory IgA antibody levels. Furthermore, it was shown that the immune response can be tailored by the co-administration of dietary molecules. The finding that the lectins used in the first study were capable of acting as carriers to prime for an anti-DNP response suggested that the potential exists for at least some of these mucosal immunogens to act as "carriers" for other antigens, therefore improving the relatively poor uptake of most antigens across the intestinal epithelium.

The following study was designed to investigate whether this carrier potential exists and to establish some of the various parameters for its successful use as a means of formulating new and more effective oral vaccines and/or orally administered drugs.

Materials and Methods

Conjugation of antigens to the mucosal immunogens

Dinitrophenylation of carriers

DNFB was reacted with Lectins and carrier as described above (see general methods)

Lipopolysaccharide (LPS) and polysaccharide (PS conjugation

S. typhimurium LPS and PS were purified as described previously (accompanying examples). LPS and PS were coupled to the MI using the periodate method (Avrameus and Ternynck, 1971).

Glutaraldehyde coupling

Leutinizing hormone releasing hormone (LHRH), bovine serum albumin (BSA) (from Sigma Chemical co. St. Louis, Miss) and S. typhimurium flagella were individually coupled to MI using the two step glutaraldehyde procedure of Ayrameus et al (1978). Briefly, the required protein was reacted with 0.2% glutaraldehyde for 2 hrs at R.T. proteins were dialysed overnight against carb/bicarb buffer pH 9.5 followed by the addition of MI at molar ratio's of 5, 10, 20 and 40:1, Antigen:MI, as required and reacted for 24 hrs at RT. Finally ethanclamine (Sigma) was added to a final concentration of 0.1M, (1 hr, RT) followd by overnight dialysis at 4C against 0.1 M carb/bicarb buffer pH 9.5.

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Peroxidase conjugated lectins

Commercial preparations of peroxidase conjugated to the lectins from Glycine max, Arachis hypogea, Tetragonolobus purpureas and conconavalin A were puchased from Sigma.

5   Chemical synthesis of LHRH conjugates

LHRH was conjugated to LTB using the glutaraldehyde procedure outlined above. Glutaraldehyde activated LHRH was added to LTB at a ratio of 20:1 LHRH:LTB and allowed to couple O/N at R.temp. The resultant conjugate was dialysed extensively against 0.1 M Carb/bicarb
10   buffer pH 9.5 before feeding. Controls consisted of LHRH or LTB which had been treated with glutaraldehyde alone.

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TABLE 3.1

Antibody response to DNP-modified mucosal immunogens.

Immunogen	Dose (ug)	Anti-DNP serum IgG	Anti-DNP Int IgA	Anti-Carrier serum IgG	Anti-Carrier Int IgA
K99	20	<4	<4	875±62	3.9±5.1
K99	100	<4	<4	1351±128	16.7±2.3
DNP6.K99	20	21±10.5	<4	64±7.2	<4
DNP18.K99	500	1024±77	42±9.6	128±27.4	76±12.9
DNP1.8.K99	500	1176±164	28±14.4	3565±192	88±21.0
987P	20	<4	<4	891±76	27.8±13.6
987P	100	<4	<4	1024±89	84±22.4
DNP6.987P	20	24±3.1	<4	147±12.2	<4
DNP25.987P	500	1024±244	14±3.1	111±34.1	68±19.2
DNP2.5.987P	500	1351±196	7±1.4	2048±166	128±38.4
LTB	20	<4	<4	1351±211	12.2±4.4
DNP2.3.LTB	20	24.3±5.6	<4	445±35	<4
LTB	100	<4	<4	891±56	4.0
DNP6BSA	20	<4	<4	<4	<4
DNP6BSA	100	<4	<4	<4	<4
Con A**	20	666±84	<4	nd	nd
PW-mitogen**	20	641±119	<4	nd	nd
L. culinaris**	20	954±48	<4	nd	nd
H. pomatia**	20	591±127	<4	nd	nd
P. vulgaris**	20	1378±110	4.8±2.3	nd	nd
G. max**	20	1529±65	3.1±6.9	nd	nd
A. hypogea**	20	1276±242	<4	nd	nd
U. europeus**	20	1583±94	<4	nd	nd

* The reciprocal of the antiserum dilution that gave on ELISA reading of 0.5 after 45 min at 37C. Each value represents the mean of 5 mice ±1 standard. deviation.

** Each lectin was substituted with 4 DNP groups

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TABLE 3.2

Carrier potential of K99 for various antigens

			Immune Response*			
			Antigen		Carrier	
Immunogen	Molar Ratio	Dose (µg)	Serum IgG	Int IgA	Serum IgG	Int IgA
K99	-	20	<4	<4	875±62	3.9±5.1
K99	·–)	100	<4	<4	1351±94	16.7±2.8
BSA-K99	1:4	20	<4	<4	<4	<4
BSA-K99	1:5	500	<4	<4	73.5±11.2	<4
BSA-K99	1:10	500	<4	<4	147±48.2	<4
BSA-K99	1:20	500	222±47	126±29.2	3809±226	84±16.6
BSA-K99	1:40	500	73±19.6	44±7.5	3176±391	64±11.9
Flag-K99	1:5	20	<4	<4	<4	<4
LPS-K99	1:1**	20	<4	<4	<4	<4
PS-K99	1:1**	20	<4	<4	229±101	5.2±2.6
BSA	-	20	<4	<4	-	-
Flagellae	-	20	<4	<4	-	-
LPS	-	20	12.1±2.7	<4	-	-
PS	-	20	<4	4.2±0.4	-	-

* See Table 3.1

** Ratio based on weight.

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TABLE 3.3

Carrier potential of 987P for various antigens

Immune Response*

Immunogen	Molar Ratio	Dose (µg)	Antigen		Carrier	
			Serum IgG	Int IgA	Serum IgG	Int IgA
987P	-	20	<4	<4	891±76	27.8±13.6
987P	-	100	<4	<4	1024±83	84±26.7
BSA-987P	1:4	20	<4	<4	84±2.6	<4
BSA-987P	1:5	500	<4	<4	256±49	<4
BSA-987P	1:10	500	29.8±7.4	4.6±2.2	675±110	<4
BSA-987P	1:20	500	306±88	122±47.6	4263±408	194±38.6
BSA-987P	1:40	500	124±47	110±38.4	4705±521	156±55
Flag-987P	1:5	20	1552±361	<4	-	-
LPS-987P	1:1**	20	<4	<4	194±28.6	<4
PS-987P	1:1**	20	<4	<4	337±96	5.2±6.8
BSA	-	20	<4	<4	-	-
Flagellae	-	20	<4	<4	-	-
LPS	-	20	12.1±2.7	<4	-	-
PS	-	20	<4	4.2±0.4	-	-

*   See Table 3.1

**   Ratio based on weight.

TABLE 3.4

Effects of altering dosage of substituted carrier on the immune response

Antigen	Dose (µg)	Immune Response (Serum IgG)*	
		Anti-BSA	Anti-Carrier
BSAO.03.K99	70	13.9±4.4	776±148
BSAO.03.K99	140	36.7±14.6	1782±174
BSAO.03.K99	280	73.5±22.1	1989±215
BSAO.05.987P	70	36±2.6	891±109
BSAO.05.987P	140	168±23.7	1552±176
BSAO.05.987P	280	337±43.7	2042±180

*   See Table 2.1

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TABLE 3.5

Effect of chemically conjugated LHRH-LTB on reproductive organs
of the female mouse*

Conjugate	Coupling procedure	Route	Dose (μg)	No Mice	Body weight	Ovaries +uterus	%Body wt O+U/tot
LHRH-LTB	glut	os/os	20	5	21.19	0.03	0.17%
LHRH-LTB	glut	os/os	50	5	21.16	0.04	0.23%
LHRH	glut	os/os	50	6	20.13	0.11	0.62%
LTB	glut	os/os	50	3	18.18	0.14	0.76%
LHRH-LTB	glut	im/im	50	6	20.16	0.16	0.97%
LHRH,LTB	mix	os/os	50	7	27.32	0.15	0.55%
LHRH,LTB	mix	im/im	50	7	30.07	0.26	0.73%
LTB	-	os/os	50	7	28.26	0.24	0.87%
-	-	os/os	-	5	19:19	0.16	0.82%

* Animals received antigen on days 0 and 14. On day 28 mice were
euthanased and their reproductive tracts weighed.

TABLE 3.6

Effect of genetically constructed LHRH conjugates on the
reproductive organs of the female mouse

Conjugate	Route	Dose (μg)	Body weight	Ovaries +uterus	%Body wt O+U/tot*
B-gal-(LHRH)3.5	im/im	20	19.47	0.10	0.49
B-gal-(LHRH)3.5	im/im	20	18.44	0.10	0.56
B-gal-(LHRH)3.5	im/im	50	19.39	0.10	0.54
LTB-(LHRH)3.5	os/os	20	19.35	0.08	0.39
LHRH	os/os	20	19.34	0.10	0.44
LHRH	im/im	20	19.39	0.15	0.76
-	im/im	-	17.74	0.08	0.46
-	os/os	-	18.63	0.11	0.60

For all intramuscular injections antigens were administered in
montanide. Im/im controls received montanide/saline mixtures.
* Animals received antigen on days 0 and 14. On day 28 mice were
euthanased and their reproductive tracts weighed.

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Genetic fusion of LHRH to B-galactosidase and LTB
CONSTRUCTION OF A PLASMID VECTOR
EXPRESING A FUSED LTB/LHRH HYBRID POLYPEPTIDE

1.   DNA Fragment Containing LTB Coding Sequences

        A Hind III fragment was obtained from a pBR322 based plasmid
containing the cloned LTB gene (Leong et al, 1985), from the plasmid
NP307 in E. coli strain RC411 (Dallus et al, 1979) which had been
modified, using a Spe I site near the stop codon for LTB, and then
mung bean nuclease digestion, using standard conditions. (Unless
specified otherwise, the conditions used for standard recombinant DNA
techniques and nucleic acid modifying enzymes are as described in
Molecular Cloning, A Laboratory Manual, Maniatis, Fritsch and
Sambrook. Cold Spring Harbor Laboratory, 1982). This eliminated the
stop codon normally found after amino acid 123 in LTB, removing 9 base
pairs of DNA and fortuitously generating a Hind III site as shown
below:

                                                 H III

    .H III   Ile Ser Met Lys ↓
        ↓

        _____LTB coding sequence ATC AGT ATG AAA GCTT
                                            121 122
          Size = 573 bp

        This fragment was ligated into the vector pUC13 (Messing, 1983)
after Hind III digestion and phosphatase treatment of the plasmid
using standard conditions. This served to place the remaining
polylinker region of pUC13, including a PstI, SalI, XbaI, BamHI, SmaI,
SstI and EcoRI sites downstream of the LTB sequence containing DNA
insert.

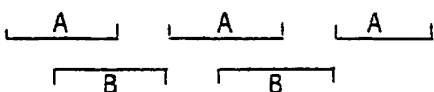2.   Creation of Synthetic LHRH Coding Oligonucleotides

        Two oligonucleotides, of 30 bases in length, with sequences
described in A and B below, were designed to form overlapping hybrid
duplexes, as shown in C, which result in a duplex which will encode
linear end to end repeats of the 10 amino acids encoding the peptide
hormone LHRH (Schally and Coy, 1983). Role of Peptides and Proteins
in Control of Reproduction, McCann and Dhindsa eds, Elsevier Science
Publishing Co, Inc. pp 89-110). In this sequence, glutamic acid
replaces the normal N-terminal pyroglutamic acid.

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A.   5'   GAG CAC TGG TCC TAC GGC CTT CGA CCC GGG   3'

B.   5'   GTA GGA CCA GTG CTC CCC GGG TCG AAG GCC   3'

C.   └─ A ─┘ └─ A ─┘ └─ A ─┘

                      ┌─ B ─┐ ┌─ B ─┐   etc.

The oligonucleotides were annealed together for 1 hr at 40°C
in 50 mM NaCl, 10 mM Tris pH 7.5, end filled with Klenow, and then the
mixture was ligated into SmaI cut M13 mp18, using standard
procedures. M13 phage containing inserts were isolated, and the DNA
sequences of the inserts were determined by the dideoxy technique.
One recombinant, designated as P29, was chosen for the fused
construct. Its DNA sequence, together with the amino acids it
encodes, in the region of the insert at the SmaI site is given below.

               N terminus of β-galactosidase, α fragment

        Met Thr Met Ile Thr Asn Ser Ser Ser Val Pro Leu Arg
        ATG ACC ATG ATT ACG AAT TCG AGC TCG GTA CCC CTT CGA
                              EcorI

        Pro Gly Glu His Trp Ser Tyr Gly Leu Arg Pro Gly
        CCC GGG GAG CAC TGG TCC TAC GGC CTT CGA CCC GGG
              ←─────────────── 1 ─────────────── →

        Clu His Trp Ser Tyr Gly Leu Arg Pro Gly Glu His Trp
        GAG CAC TGG TCC TAC GGC CTT CGA CCC GGG GAG CAC TGG
              ←─────────────── 2 ───────────────→ ←──────────

        Ser Tyr Gly Leu Arg Pro Gly Gly Asp Pro Leu Glu Ser Thr
        TCC TAC GGC CTT CGA CCC GGG GGG GAT CCT CTA GAG TCG ACC
        ──── 3 ──────────────────→   HincII

The DNA sequence confirmed the insertion of DNA from the
synthetic oligonucleotides to place an in-frame fusion of
approximately 3 and a half repeats of the LHRH encoding sequence (34
amino acids). The complete LHRH coding blocks are indicated by the
arrows.

The replicative from DNA of P29 was digested with EcoRI and
HincII (sites indicated in DNA sequence above), and end filled using
standard conditions. The small 140 base pair fragment was isolated
from a polyacrylamide gel.

3.   Construction of the LTB/LHRH Fusion Vector

The pUC13 plasmid containing the LTB coding sequence inserted in

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the HindIII site (section 1 above) was digested with SalI, end filled and phosphatased using standard conditions. The vector DNA was then ligated to the end filled EcoRI/HincII fragment from P29 (Section 2 above). The fusion should have the amino acid sequence below.

aa1 - 122 (lys)-ala-trp-ala-ala-gly-arg-asn-ser-ser-ser-val- pro-
LTB coding sequence.

leu-arg-pro-gly-[glu-his-trp-ser-tyr-gly-leu-arg-pro-gly]$_3$-
                                    LHRH

gly-asp-pro-leu-glu-ser-arg-leu

There is a stop codon 24 bases beyond the LHRH sequence which defines the production of a 176 amino acid polypeptide, with 122 amino acids from LTB, 34 amino acids encoding the LHRH repeat, and an additional 20 amino acids derived from the adjoining regions.

The correct construct was screened by digesting DNA from minipreps with Eco RI and looking for plasmids with the appropriately larger Eco RI fragment. The putative positives were further screened for the expression of this polypeptide, driven from the lac promoter of pUC13. Bacterial extracts were analysed on polyacrylamide gels followed by transfer to nitrocellulose paper and Western blotting with rabbit antisera directed against an LTB and LHRH conjugate. A peptide of the expected size was detected by both antisera.

4.   Construction of expression plasmid K66 and expression strain
      BTA1185

A 573 bp Eco RI fragment of the pUC13 LTB-LHRH fusion plasmid described in 3, which contains the LTB-LHRH fusion coding region in its entirety, was isolated from an agarose gel and ligated into Eco RI cut, phosphatased expression vector pKK223-3 (from Pharmacia). The resultant expression plasmid PBTA K66, placed the expression of the fusion protein under the control of the tac promoter, where expression is induced with IPTG. The plasmid was transformed into E. coli host strain JM101 (SupE, thi, (lac-pro AB) [F' traD36, pro AB lacI^q Z M 15) to give the host vector expression system BTA1185.

5.   Production and Purification of LTB/LHRH Fusion Protein for
      Animal Trials.

The LTB(LHRH)$_{3.5}$ producing strain was grown as described

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previously. After induction with IPTG for 2 h bacteria were pelleted
by centrifugation (3,000 x g, 10 min at 4°C). Bacteria were then
resuspended in dH_2O and lysed in a French Press. After removal of
the bacterial debris by centrifugation (18,000 x g, 10 min, at 4°C)
the supernatant was loaded onto an agthio-galactose column (Sigma).
The fusion protein was then eluted with 0.5M galactose and dialysed
against 0.1M carb/bicarb buffer pH 9.5.

Antigen adminstration and measurement of the immune response

All oral presentation procedures, antibody collections and ELISA
determinations were as described previously.

RESULTS

Demonstration of the carrier potential of the mucosal immunogens

All of the mucosal immunogens tested showed the capacity to
effectively transport the covalently attached hapten DNP across the
intestinal mucosa and to elicit a serum anti-DNP Ab response after
feeding of the dinitrophenylated-MI. DNP-modified BSA, however was
completely ineffective in eliciting an anti-DNP or anti-BSA response
when fed at the concentrations tested (Table 3.1). Initial
experiments in which K99 and 987P were complexed to much larger
molecules than DNP were unsuccessful in generating immune responses to
either the mucosal immunogen or to the molecule coupled to it (Tables
3.2 & 3.2), possibly due to steric interference in the binding of the
pili to the mucosal epithelium. It was therefore decided to vary the
ratio of antigen to MI. When various ratios of BSA:pili were tested,
it was found that when ratios of greater than 1:20 BSA:pili were fed
it was not possible to generate either anti-BSA or anti-pili responses
even with a dose of 500 ug, demonstrating that it was not possible for
the complexes to effectively associate with ther mucosal epithelium
and to therefore generate an immune response. However when ratios of
1:20 or 1:40 were employed good responses to both BSA and to pili were
observed (Tables 3.2 & 3.3). The magnitude of the immune response was
readily varied by altering the doses of complex fed (Table 3.4).

Oral administration of LHRH coupled to LTB lead to a significant
reduction in the combined uterine and ovarian weights of female mice
receiving either 20 or 50 ug LHRH-LTB (P 0.05) (Table 3.5,3.6). No
such weight loss was seen with either LHRH or LTB fed alone or
together or to intramuscular injection of LHRH-B-galactosidase,

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LHRH-LTB, or free LHRH. The effect of the weight loss was also seen developmentally as there was a complete absence of mature follicules in the ovaries, thus, the animals were effectively "castrated". There was a slight reduction in reproductive tract weights when mice were fed the genetically constructed LTB-(LHRH)$_{3.5}$ fusion protein (Table 3.6) but in this experiment, the reduction was not significant at the doses tried.

EXAMPLE 4

Induction of cell-mediated immunity after oral administration of antigen

Feeding of mucosal immunogens was shown to be effective in eliciting humoral responses as measured by the production of serum and intestinal antibodies. It was not known, however whether there was a concomitant stimulation of a cell mediated immune (CMI) response to the mucosal immunogens.

The following study was designed to compare the CMI generated by oral presentation of a mucosal immunogen with that generated by classic subcutaneous (s.c.) injection of antigen in Complete Freund's Adjuvant (CFA).

Methods

Male C57B1/6J mice were immunized by feeding 20 µg antigen in 0.1 M carb/bicarb buffer pH 9.65 or by injecting 20 µg antigen in CFA s.c. Controls received only buffer or adjuvant. Seven days after immunization mice were injected in the left back footpad with 10 µg of antigen in 20 µl saline, and injected with 20 µl saline alone in the right rear footpad. After a further 24 hours, the difference in thickness between the left and right rear footpads was measured using a micrometer.

Results

The results shown in table 4.1 demonstrates that a good cellular immune response is generated upon oral feeding of either 987P or LTB mucosal immunogens. In fact the response was suprisingly high as it was only slightly smaller than that generated by the s.c. injection of these antigens in CFA.

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Table 4.1

Generation of a cell mediated immune resonse upon oral
presentation of mucosal immunogens.

	Immunisation route		
Antigen	Control	Oral	Subcutaneous
987P	11 ± 2	31 ± 6.6	38 ± 5
LTB	5 ± 4.5	14 ± 3.7	26 ± 5.8

*   Results represent the increase in footpad size after
immunization with antigen. Results are the mean of 5 mice ± standard
deviation.

INDUSTRIAL APPLICABILITY

Industrial applications of the invention include the
preparation of oral medicaments for administration to vertebrate hosts.

Potential Vaccine Candidates for Oral Vaccine

Allergens: Various Grass Pollens: barley, couch
                          Weed Pollens: clover, dock
                          Tree Pollens: ash, cyprus
                          Plant Pollens: broom   -
                          Epithelia: car hair, dog hair, pig hair
                          Miscellaneous: house dust, wheat chaff, Kapok.
Hormones: LHRH, FSH, HGH, Inhibin
Vaccines: Haemagglutunins from
                          Influenza, Measles, Rubella, Smallpox, Yellow
                          Fever, Diphtheria, Tetanus, Cholera, Plague,
                          Typhus, BCG, Haemophilus influenzae, Neisseria
                          catarrhalis, Klebsiella pneumoniae,
                          Pneumococci, streptococci esp. S. mutans.
Pili from: E. Coli, N. gonorrhoeae, N. meningitis, N.
                          catarrhalis, Yersinia spp., Pesudomonas
                          aeruginosa, Pseudomonas spp., Moraxella bovis,
                          Bacteroides nodosus, Staph spp., Strep spp.,
                          Bordetella spp.?

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175

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CLAIMS

1.   A complex comprising: an immunogen linked to a carrier molecule which carrier molecule is capable of specifically interacting with the mucosal epithelium of a vertebrate host; wherein both the immunological activity of the immunogen and the capacity of the carrier molecule to specifically interact with the mucosal epithelium of the vertebrate host is substantially maintained, and said complex is capable of eliciting a systemic, cellular and/or mucosal immune response in the vertebrate host.

2.   A complex according to claim 1 wherein said immunogen is selected from; all, part(s), an alalogue, homologue, derivative or combination thereof of a hormone, therapeutic agent, antigen or hapten.

3.   A complex according to claim 1 wherein said complex is capable of eliciting a systemic or cellular immune response in the vertebrate host.

4.   A complex according to claim 1 wherein said complex is capable of eliciting a mucosal immune response in the vertebrate host.

5.   A complex according to any one of claims 1 to 4 wherein the immunogen is all, part, an analogue, homologue, derivative or combination thereof of an antigen or hapten.

6.   A complex according to any one of claims 1 to 5, wherein the immunogen is all, part, an analogue, homologue, derivative or combination thereof of a hormone.

7.   A complex according to any one of claims 1 to 6 wherein the immunogen is all, part, an analogue, homologue, derivative or combination thereof, of luteinising hormone releasing hormone.

8.   A complex according to any one of claims 1 to 6 wherein the immunogen is FSH, HGH, inhibin.

9.   A complex according to any one of claims 1 to 5, wherein the immunogen is an allergen.

10.   A complex according to claim 9, wherein the allergen is a grass pollen, weed pollen, tree pollen, plant pollen, cat hair, dog hair, pig hair or other epithelia or house, dust, wheat chaff or kapok.

11.   A complex according to any one of claims 1 to 4, wherein the immunogen is a surface protein derived from influenza, measles, Rubella, smallpox, yellow fever, diphtheria, tetanus, cholera, plague, Typhus or BCG causing agents, Haemophilus influenzae, Neisseria

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catarrhalis, Klebsiella pneumoniae, pneumococci and streptococci; or a
pilus derived from E.coli, N.gonorrhoeae, N.neningitidis,
N.catarrhalis, Yersinia spp, Pseudomonas aeruginosa, Pseudomonas spp,
Moraxella bovis, Bacteroides nodosus, Staphylococci spp, Streptococci
spp and Bordetella spp.

12.   A complex according to any one of claims 1 to 4, wherein
the immunogen is a surface polysaccharide derived from diphtheria,
tetanus, cholera, plague, Typhus or BCG causing agents, Haemophilus
influenzae, Neisseria catarrhalis, Klebsiella pneumoniae, pneumococci
and streptococci; or a pilus derived from E.coli, N.gonorrhoeae,
N.neningitidis, N.catarrhalis, Yersinia spp, Pseudomonas aeruginosa,
Pseudomonas spp, Moraxella bovis, Bacteroides nodosus, Staphylococci
spp, Streptococci spp and Bordetella spp.

13.   A complex according to any one of claims 1 to 4, wherein
the immunogen is a secretory product derived from diphtheria, tetanus,
cholera, plague, Typhus or BCG causing agents, Haemophilus influenzae,
Neisseria catarrhalis, Klebsiella pneumoniae, pneumococci and
streptococci; or a pilus derived from E.coli, N.gonorrhoeae,
N.neningitidis, N.catarrhalis, Yersinia spp, Pseudomonas aeruginosa,
Pseudomonas spp, Moraxella bovis, Bacteroides nodosus, Staphylococci
spp, Streptococci spp and Bordetella spp.

14.   A complex according to any one of claims 11 to 13 wherein
the surface protein, polysaccharide or secretory product is derived
from Streptococcus mutans.

15.   A complex according to any one of claims 1 to 14 wherein
the carrier molecule is all, part, an analogue, homologue, derivative
or combination thereof of a bacterial adhesin, viral haemagglutinin, a
toxin or the binding subunit thereof, or a lectin whether of plant or
other origin.

16.   A complex according to claim 15 wherein the carrier
molecule is all, part, an analogue, homologue, derivative or
combination thereof of the heat labile toxin of enterotoxigenic E coli.

17.   A complex according to claim 15 wherein the carrier
molecule is all, part, an analogue, homologue, derivative or
combination thereof of a bacterial pilus.

18.   A complex according to claim 17 wherein the bacterial pilus
is the K99 or 987P pilus of E coli.

19. A complex according to claim 17 wherein the bacterial pilus
is CFAI, CFAII, K88 or F41.

20. A complex according to claim 15 wherein the carrier
molecule is all, part, an analogue, homologue, derivative or
combination thereof of a lectin.

21. A complex according to claim 20 wherein the lectin is
Conconavlin A, Pokeweed mitogen or the lectin from Lens culinaris,
Helix pomatia, Glycine max, Arachis hypogea, or Ulex europeus.

22. A complex according to claim 20 wherein the lectin is
Abrin, Asparagus pea, Broad bean, Camels foot tree, Castor bean, Fava
bean, Green marine algae, Hairy vetch, Horsegram, Horseshoe crab, Jack
bean, Japanese wisteria, Jequirity bean, Scotch Laburnum, Lima bean,
Limulin, Lotus, European Mistletoe, Mung bean, Osage orange, Pogada
tree, Garden pea, Potato, Red kidney bean, Red marine algae, Siberian
pea tree, edible snail, garden snail, Spindle tree, Sweet pea, Tomato,
Wheat germ or Winged pea lectin.

23. A complex according to claim 15, wherein the carrier
molecule is all, part, an analogue, homologue, derivative or
combination thereof of a viral haemagglutinin.

24. A complex according to claim 23 wherein the viral
haemagglutinin is a haemagglutinin derived from influenza, measles,
Rubella, smallpox or yellow fever virus.

25. A complex according to any one of claims 1 to 4 wherein the
immunogen is all, part, an analogue, homologue, derivative or
combination thereof of LHRH and the carrier molecule is all, part, an
analogue, homologue, derivative or combination thereof of LTB.

26. A process for the production of a complex according to any
one of claims 1 to 25 which process comprises:

(a) reacting the immunogen with the carrier molecule to form
said complex;

(b) chemically modifying the immunogen to provide at least one
functional group capable of forming a chemical linkage, and reacting
the immunogen and carrier molecule to form said complex; or

(c) chemically modifying the carrier molecule to provide at
least one functional group capable of forming a chemical linkage, and
reacting the immunogen and carrier molecule to form said complex;

(d) chemically modifying the immunogen and the carrier molecule

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to provide functional groups capable of forming a chemical linkage, and reacting the immunogen and carrier molecule to form said complex;

(e)   reacting the immunogen with at least one linking agent, and reacting the immunogen and the carrier molecule to form said complex;

(f)   reacting the carrier molecule with at least one linking agent and reacting the immunogen and the carrier molecule to form said complex;

(g)   reacting the immunogen and the carrier molecule with at least one linking agent, and reacting the immunogen and the carrier molecule to form said complex; or

(h)   a combination of any of the preceding process steps.

27.   A process for the production of a complex according to any one of claims 1 to 25 which process comprises:

providing a recombinant DNA molecule comprising a first DNA sequence which on expression codes for the amino acid sequence of the immunogen, a second DNA sequence which on expression codes for the amino acid sequence of the carrier molecule, and vector DNA; transforming a host with said recombinant DNA molecule so that said host is capable of expressing a hybrid, proteinaceous product which comprises said complex; culturing said host to obtain said expression; and collecting said hybrid proteinaceous product.

28.   A process for the production of a complex according to any one of claims 1 to 25 which process comprises:

(a)   chemically synthesizing the immunogen and/or the carrier molecule, and forming the complex by reactions according to the process of claim 26;

or   (b)   synthesizing a hybrid peptide comprising amino acid sequences of the immunogen and the carrier molecule.

29.   A process according to claim 28 wherein the peptide is prepared by solid phase, enzymatic or manual peptide synthesis.

30.   A process according to claim 29 wherein the peptide is prepared by solid phase peptide synthesis.

31.   A process according to claim 30 wherein the synthesized immunogen or carrier molecule whilst bound to the resin of the solid phase peptide synthesizer is coupled to the carrier molecule or immunogen respectively.

32.   A process according to any one of claims 28 to 31 wherein

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the synthetic peptide is all, part, an analogue, homologue, derivative or combination thereof of LHRH.

33. A process according to claim 26 wherein the carrier molecule is LTB and the linking agent is glutaraldehyde.

34. A complex according to any one of claims 1 to 25 when made by a process according to any one of claims 26 to 31.

35. An expression product of a transformant host which comprises a complex according to any one of claims 1 to 25.

36. A transformant host transformed with a recombinant DNA molecule comprising a first DNA sequence which on expression codes for the amino acid sequences of all, part, an analogue, homologue, derivative or combination thereof, of the immunogen, a second DNA sequence which on expression codes for the amino acid sequence of all, part, an analogue, homologue, derivative or combination thereof of the carrier molecule, and vector DNA.

37. A transformant host according to claim 30 wherein said host a Gram negative bacterium, a Gram positive bacterium, a yeast, fungus, or a higher eukaryotic cell.

38. A transformant host according to claim 36 or claim 37 wherein said host is E.coli.

39. A culture of the transformant microorganism designated: ATCC

40. A recombinant DNA molecule comprising a first DNA sequence which on expression codes for the amino acid sequence of the immunogen, a second DNA sequence which on expression codes for the amino acid sequence of the carrier molecule, and vector DNA.

41. A recombinant DNA molecule according to claim 40 wherein vector DNA is plasmid DNA.

42. Plasmid pBTAK66.

43. A recombinant DNA molecule according to claim 40 wherein vector DNA is viral, bacteriophage or cosmid DNA.

44. A polynucleotide sequence which comprises a first hybrid nucleotide sequence which acts as a coding sequence for a fusion protein comprising an amino acid sequence of an immunogen fused to an amino acid sequence of a carrier molecule, a polynucleotide sequence sufficiently related to said first hybrid polynucleotide sequence so as to hybridize to said first hybrid polynucleotide sequence, a polynucleotide sequence related by mutation, including single or

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multiple base substitutions, deletions, insertions and inversions, to
said first hybrid polynucleotide sequence or hybridizing sequence or a
polynucleotide sequence which on expression codes for a polypeptide
which displays similar biological or immunological activity to said
fusion product.

45.   A polynucleotide sequence according to claim 44 wherein the
first hybrid polynucleotide sequence acts as a coding sequence for the
amino acid sequence of all, part, an analogue, homologue, derivative
or combinations thereof,) of an antigen or hapten fused to the amino
acid sequence of a carrier molecule.

46.   A polynucleotide sequence according to claim 44 wherein the
first hybrid polynucleotide sequence acts as a coding sequence for the
amino acid sequence of all, part, an analogue, homologue, derivative
or combinations thereof of LHRH fused to the amino acid sequence of a
carrier molecule.

47.   A medicament which comprises a complex according to any one
of claims 1 to 25 together with a pharmaceutically acceptable carrier
or diluent.

48.   A medicament according to claim 47 adapted for oral
administration.

49.   A medicament according to claim 47 adapted for nasal
administration.

50.   A medicament according to any one of claims 47 to 49
wherein said medicament is in capsule, tablet, slow release, elixir,
gel, paste or nasal spray form.

51.   A medicament according to any one of claims 47 to 50 which
additionally comprises a dietary molecule which dietary molecule can
selectively modulate the magnitude and/or type of immune response to
the immunogen of the medicament.

52.   A medicament according to claim 51 wherein the dietary
molecule is selected from amino acids, vitamins, monosaccharides and
oligosaccharides.

53.   A medicament according to claim 51 or claim 52 wherein the
dietary molecule is selected from vitamin A, vitamin B_1, vitamin
B_2, Vitamin B_6, Vitamin B_{12}, Vitamin C, Vitamin D, Vitamin E,
fructose, lactose, mannose, melibiose, sorbitol or xylose.

54.   A method of presenting a complex according to any one of

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INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 86/00135

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.[4]   A61K 39/385, C07K 17/00, C12N 1/20, 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC:	A61K 39/385, 39/39, 39/44, 47/00, C07G 7/00, 17/00, C07K 7/20, 13/00, C12N 1/20, 15/00, C07C 103/52

Chemical Abstracts keywords:pili, Fimbriae, vaccine

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU: IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No [13]
X	US, A, 4 496 538 (L.K. LANCE) 29 January 1985 (29.01.85)	1-5, 11, 12, 15, 26, 28, 47
X,P	AU, A, 44588/85 (PRAXIS BIOLOGICS INC) 13 February 1986 (13.02.86) see pages 8, 9, 14, 26, 27	1,3-5, 9, 11, 12, 13, 15-19, 26, 33-34,47
X,P	WO, A, 85/05037 (SYNTEK) 21 November 1985 (21.11.85)	1-5,11,17-19 26,27,35-38 40,41, 43-45 47-50, 55, 56
X,P	WO, A, 85/04654(SCRIPPS CLINIC AND RESEARCH FOUNDATION) 24 October 1985 (24.10.85)	1,3-5, 11, 15, 17,26-31, 34-38, 40-41 43-50, 54-56
X	WO, A, 83/03971(PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 24 November 1983 (24.11.83)	1-5, 11-13, 15 26, 27, 35-38, 40, 41, 43-45 47

continued

* Special categories of cited documents [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 August 1986 (13.08.86)	26 AUGUST 1986 (26.08.86)
International Searching Authority	Signature of Authorized Officer
AUSTRALIAN PATENT OFFICE	J.H. CHAN

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

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X	US, A, 4372883 (MATUHASHI et al) 8 February 1983 (08.02.83)	1-6
X	Journal of Immunological Methods, Volume 48 issued 1982 (Amsterdam, Netherlands) P.R. Lambden and J.E. Heckels, 'Synthesis of Immunogenic oligosaccharide-Protein Conjugates from Lipopolysaccharides of <u>Neisseria</u> <u>gonorrhoeae</u> P 9', see pages 233-240	1-5, 1, 12, 15, 17, 26, 47
X	EP, A, 0 116 201 (CHIRON CORP) 22 August 1984 (22.08.84) see page 2 line 11 - page 3 line 16	40, 41, 43 44
X	WO, A, 83/02393 (ANIMAL RESEARCH CORPORATION) 21 July 1983 (21.07.83)	40, 43
X	GB, A, 2071671 (HARVARD COLLEGE PRESIDENT AND FELLOWS OF CAMBRIDGE) 23 September 1981 (23.09.81)	40, 41, 43 44
X	EP, A, 0 020 147 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 10 December 1980 (10.12.80) see page 2 line 5 - page 3 line 6, page 11 line 36 - page 12	40, 41, 43, 44
X	EP, A, 0 049 945 (BACTEX INC) 21 April 1982 (21.04.82)	63
X	GB, A, 2 094 314 (THE WELLCOME FOUNDATION LTD) 15 September 1982 (15.09.82)	63
X	GB, B, 1 529 871 (DORNER et al) 25 October 1978 (25.10.78)	63
Y	AU, A, 34497/84 (AKZO N.V.) 26 April 1985 (26.04.85) See pages 2-9	1-3, 5-7, 11-12, 15, 25-26, 28, 32, 34, 48-50
Y	US, A, 4556555 (K.L. ESBENSHADE) 3 December 1985 (03.12.85)	7, 25, 57
Y	AU, A, 11990/83 (CYTOGEN CORPORATION) 15 September 1983 (15.09.83)	26
Y	Progress in Allergy, Volume 33, issued 1983 (Basel, Switzerland) Schoolnik <u>et al</u>, 'A Pilus Peptide Vaccine for the Prevention of Gonorrhea', see pages 314-331	1-63
X, Y	M. Sussman, 'The Virulence of Escherichia coli' 1985, Academic Press, see pages 82-37, 111-119	63

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members					
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AU 44588/85	EP 172107	WO	8601228			
WO 8505037	AU 42994/85					
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WO 8303971	AU 17062/83	EP	108146	ES	522315	
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	DK 2630/76	ES	448827	FR	2313942	
	GB 1553664	IT	1078623	JP	51151322	
	NL 7606379	US	4136167	ZA	7603370	
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	GB 2061955					
EP 116201	DK 5778/83	JP	59132892			

Patent Document Cited in Search Report	Patent Family Members					
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	IN	156614	NO	833205	NZ	202954
	PT	76079	US	4593002		
GB 2071671	AT	1222/81	BE	887991	CA	1198385
	DE	3110031	ES	500397	ES	510007
	FR	2478125	JP	56167698	NL	8101289
	SE	8101665				
EP 20147	AU	58926/80	CA	1194432	DD	202045
	DK	2329/80	EG	15156	EP	20147
	ES	492044	ES	499045	FI	801743
	IL	60186	JP	56036499	NZ	1939867
	PL	224664	PT	71323	YU	1453/80
	ZA	8002992				
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GB 1529871	AT	8293/75	AU	86282/75	BE	835075
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	FR	2289206	IL	58384	JP	51070810
	NL	7512590	NO	753578	NZ	179108
	SE	7511907	SE	8100404	ZA	7506873
AU 34497/84	EP	142192	JP	60172932	JP	60172933
AU 11990/83	CA	1203164	DK	1130/83	EP	88695
	JP	5822035	ZA	8301463		

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              1              5              10
Type 1    Ala-Ala-Thr-Thr-Val-Asn-Gly-Gly-Thr-Val-His-Phe-Lys-Gly-

K88       Trp-Met-Thr-Gly-Asp-Phe-Asn-Gly-Ser-Val-Asp-Ile-Gly-Gly-

K99       Asn-Thr-Gly-The-Ile-Asn-Phe-Asn-Gly-Lys-Ile-Thr-Ser-Ala-

987P      Ala-Pro-Val-Glu-Asn-Asn-Thr-Cys-Gln-Ala-Asn-Leu-Asp-Phe-

Neisseria Phe-Thr-Leu-Ile-Glu-Leu-Met-Ile-Val-Ile-Ala-Ile-Val-Gly-

              15             20             25
Type 1    Glu-Val-Val-Asn-Ala-Ala-

K88       Ser-Ile-Thr-Ala-Asp-Asp-Tyr-Arg-

K99       Thr-Cys-Thr-Ile-Glu-Pro-Glu-Ala-

987P      Thr-Gly-Lys-Val-Thr-Ala- x -Leu-

Neisseria Ile-Leu-Ala-Ala-Val-Ala-Leu-Pro-
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Fig.1   N-terminal amino acid sequence of the 987P pilin subunit. The
         N-terminal sequences of other pilin proteins are given for
         comparison.

SUBSTITUTE SHEET

claims 1 to 25 to a vertebrate host which method comprises the mucosal administration of a medicament according to any one of claims 47 to 51.

55. A method of presenting a complex according to any one of claims 1 to 25 to a vertebrate host which method comprises the oral administration of a medicament according to any one of claims 47, 48, 50 and 51.

56. A method of presenting a complex according to any one of claims 1 to 25 to a vertebrate host which method comprises the nasal administration of a medicament according to any one of claims 47, 49, 50 or 51.

57. A method of inhibiting the gonadal function in a mammal, which method comprises the mucosal administration of a medicament according to any one of claims 47 to 51.

58. A method of selectively modulating the magnitude and/or type of immune response to the immunogen of a complex according to any one of claims 1 to 25 which method comprises the mucosal administration of a medicament according to claim 51 to a host vertebrate.

59. A method of selectively modulating cellular immune responses to a complex according to any one of claims 1 to 25 which method comprises the mucosal administration of a medicament according to claim 45 in such a way as to specifically increase or decrease the cellular immune response to the complex in the vertebrate host.

60. A method according to claim 58 or claim 59 which comprises the administration of a medicament according to any one of claims 47 to 50 and a dietary molecule.

61. A complex substantially as hereinbefore described with reference to the Examples.

62. A method for the induction or modulation of an immune response in a host substantially as hereinbefore described with reference to the Examples.

63. A carrier molecule substantially as hereinbefore described wih reference to the Figure.